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Galanin is an autocrine myelin and oligodendrocyte trophic signal induced by Leukemia inhibitory Factor

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Running title: LIF induces galanin release in oligodendroglia

Main Points:

- Three known trophic factors for oligodendrocytes (LIF, NT3, Insulin) induce largely non-overlapping gene expression changes in post-mitotic oligodendrocytes (OC)
- LIF induces the synthesis and secretion of galanin in OC in vivo and in vitro
- LIF-induced OC survival is mediated via auto/paracrine galanin secretion

Key Words: Leukemia Inhibitory Factor, Galanin, Oligodendrocytes

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Abstract

In order to further investigate the molecular mechanisms that regulate oligodendrocyte (OC) survival, we utilized microarrays to characterize changes in OC gene expression after exposure to the cytokines neurotrophin3, insulin or leukemia inhibitory factor (LIF) *in vitro*. We identified and validated the induction and secretion of the neuropeptide galanin in OCs, specifically in response to LIF. We next established that galanin is an OC survival factor and showed that autocrine or paracrine galanin secretion mediates LIF-induced OC survival *in vitro*. Using the cuprizone model of demyelination, we showed that galanin is up-regulated in OCs in this animal model of central demyelination and that oligodendroglial galanin expression is significantly regulated by endogenous LIF in this context. We also showed that knock-out of galanin reduces OC survival and exacerbates callosal demyelination in the cuprizone model.

These findings suggest a potential role for the use of galanin agonists in the treatment of human demyelinating diseases.

Introduction

The oligodendrocyte (OC) is the specialized glial cell within the central nervous system (CNS) that is responsible for producing myelin and is targeted in central demyelinating disease. Therefore, an understanding of the molecular mechanisms regulating OC survival and myelin maintenance could inform novel therapeutic strategies.

A limited number of growth factor families, including the neurotrophins, insulin and insulin-like growth factors, as well as members of the interleukin-6 family, are known to enhance OC survival, and generally do not act synergistically (Barres et al. 1992; Barres et al. 1993; Casaccia-Bonnet 2000; Kahn and De Vellis 1994). The molecular mechanisms by which these factors influence OC survival are incompletely understood. On the one hand, there is considerable evidence that a number of key signaling pathways are activated in response to receptor binding and activation by these cytokines, including the signal transducer and activator of transcription (STATs) 1 and 3 (Boulton et al. 1994; Sadowski et al. 1993); Akt (Alonzi et al. 2001) and Mek/MAPK pathways (Ernst et al. 1999; Tomida et al. 1999). It remains uncertain, however, whether these signaling pathways are used in a generic sense by each of the relevant growth factor families in order to promote OC survival, or whether individual growth factor families ultimately act by disparate mechanisms.

In this study, we examined gene expression changes in OCs following incubation with three cytokines known to promote their survival, namely insulin (Barres et al. 1992; 1993), neurotrophin-3 (NT3, Barres et al. 1993), and the interleukin-6 family member leukemia inhibitory factor (LIF, Barres et al. 1993). Little concordance was found between genes induced by LIF, insulin or neurotrophin-3. Interestingly, one response unique to LIF was the induction of galanin, a neuropeptide with known trophic and survival roles in neurons.

Galanin is a 29 amino acid neuropeptide that was originally isolated from porcine intestine. Under normal basal physiological conditions, galanin is synthesized and secreted by a number of neuronal populations, where it predominantly functions as an inhibitor of synaptic transmission. The functions of galanin in OCs are not well defined, but we have previously shown that galanin is exclusively up regulated in OCs in acute lesional tissue using the murine MOG₃₅₋₅₅ experimental autoimmune encephalomyelitis model (EAE) (Wraith et al. 2009). Furthermore, in the same study, using galanin-knockout (Gal-KO) and galanin-overexpressing transgenic mice we showed that depletion of endogenous galanin expression markedly worsens disease outcome in EAE, whereas galanin overexpressing transgenics exhibited abrogated disease. A recently published study also suggests that transgenic mice with increased systemic galanin levels due to galanin overexpression in the anterior pituitary are relatively resistant to cuprizone-mediated callosal demyelination and exhibit a 2.5 fold increase in GST-pi positive callosal OC numbers at the time of maximal demyelination (Zhang et al. 2012). It remains unclear however if the transgenic systemic overexpression of galanin acts directly on OCs to promote these beneficial effects.

Using primary OC cultures from Gal-KO and wildtype mice, we investigate if galanin, itself, acts as a survival factor for OCs. Furthermore, in juxtaposition to previous studies, we investigate if an absence of endogenous galanin increases the

severity of demyelination and OC loss induced by systemic cuprizone ingestion, to determine if the upregulation of galanin in wild-type OC is also associated with reduced disease-related demyelination *in vivo*.

Materials and Methods:

All experiments were conducted under the auspices of and were approved by the Howard Florey Institute (HFI) Animal Ethics Committees and in fulfilment of the National Health and Medical Research Council of Australia Animal Research guidelines.

Mice

The Gal-KO mice on the 129OlaHsd background were as previously described (Wynick et al. 1998), the PLP-dsRED transgenic mice were generated by Dr Frank Kirchhoff (backcrossed 12 generations to C57Bl6 in our laboratory) and the LIF-knockout and CNTF-knockout mouse lines on the C57Bl6 background were a kind gift from Professor Michael Sendtner, Wuerzburg (Bugga et al. 1998; Masu et al. 1993). Littermate wild-type controls were used for all cuprizone experiments. For cuprizone-induced demyelination, 9-week-old mice were fed ad libitum a diet of powdered feed (Barastoc; Ridley AgriProducts, Pakenham, Victoria) mixed with cuprizone (Sigma-Aldrich) at 0.2% (w/w) for 18 days. At experimental end-point, the mice were killed by intraperitoneal injection of pentobarbitone.

For immunohistochemistry processing, mice were transcardially perfused with 20 mL of PBS, followed by 20 mL of 4% paraformaldehyde (PFA) solution. The brains were dissected out and postfixed in 4% PFA solution overnight and then cryoprotected in 20% sucrose overnight prior to blocking in Tissue-Tek (Sakura). Serial 10 µm coronal sections were cut on a cryostat (Leica Microsystems).

For electron microscopic evaluation of caudal callosal myelinated axon numbers, mice were transcardially perfused with 4% PFA/2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. The caudal callosal region was dissected and post-fixed in 2% Osmium tetroxide/1.5% potassium ferricyanide then routinely processed for EM and embedded in Spurr's resin. Samples were sectioned with a Reichert Ultracut S ultramicrotome for cross-sections at 0.5 µm for methylene-blue staining. For each callosal section examined, three images from non-overlapping areas of the corpus callosum corresponding to approximately Bregma 0.38 were acquired at 100 times magnification on a Zeiss Axioplan 2 Imaging system (Axiovision Software Release 4.4), and 17400 µm² sections from the centre of each image were used to count the number of myelinated axons (see Fig 6). The three individual counts per animal were averaged and expressed as the number of myelinated axons/section.

Rat astrocyte cell cultures

Primary mixed glial cultures were prepared from the cortices of P1-2 Sprague-Dawley rat pups as previously described (McCarthy and de Vellis 1980, modified by Binder et al, 2008). . Once grown to confluence (day 7), microglia and OCs were removed from the mixed glial culture by shaking the culture at 100 rpm for two hours and 180 rpm overnight respectively. Astroglial cultures were further purified by gentle percussion to achieve a purity of >95%. Astrocytes were maintained in DMEM 10. For passaging, purified astrocytes were trypsinized (1:1000 of 2.5% stock, Stratagene) for 8 minutes at 37°C/5% CO₂. The reaction was stopped with the addition of

DMEM10. Cells were counted using a haemocytometer and plated in appropriate PDL-coated 10 cm dishes for experimentation.

Oligodendrocyte cell cultures

We purified rat OCs from P6 rat optic nerve oligodendrocyte precursor cells (OPCs) as previously described (Barres et al. 1992; Binder et al. 2008).

Mouse OPCs were isolated from P6 mouse cortex like their rat counterparts (Cahoy et al. 2008), with the following modifications. The cells were immunopanned over three sequential Anti BSL1/Griffonia simplicifolia Lectin 1 (Vector Labs L-1100) coated plates followed by positive selection with an anti-PDGFR α antibody (Research Diagnostics) coated plate, and the rat SATO was modified by inclusion of 20 μ l/ml of B27 (Gibco)

RNA preparation for Affymetrix arrays and real-time PCR

For the gene array experiments, the cells were washed twice with SATO then placed in SATO (without growth factors, or with 5ng/mL NT3, or 5 μ g/mL Insulin, or 100ng/ml LIF) for 24 hours before harvesting. For real-time PCR validation, rat OC were plated in SATO and exposed for either 8 or 24 hrs to LIF (100ng/ml) or insulin (5 μ g/mL).

For both array and real-time PCR experiments, OC RNA was then isolated using the Qiagen RNeasy Mini Kit (Animal cell –I) according to the manufacturer's instructions. Ten μ g of total RNA was subjected to DNase treatment to remove any remaining DNA and the Qiagen RNeasy Mini Kit (RNA Cleanup) was used to purify RNA that was eluted in a final volume of 30 μ l in RNase-free water. RNA concentration and purity were quantified using a Nanodrop spectrophotometer (Biolab, Australia).

Affymetrix Micro Array

For micro array analysis RNA from three independent biological replicate experiments were harvested for each treatment. One μ g of total RNA was reverse transcribed using the 3' IVT Express Kit (Affymetrix) and hybridized to the Rat Genome 230 2.0 GeneChip (Affymetrix) using the GeneChip Hybridization, Wash, and Stain Kit (Affymetrix) according to manufactures instructions at the Australian Genome Research Facility (AGRF, Parkville). Microarray data was analysed using Partek[®] genomics suite version 6.5 (6.3) (Partek Inc, St Louis, MO, USA). One control treatment sample was excluded as it was identified as an outlier during quality control analysis of the expression data. An ANOVA model, including both the treatment and replicate groups, was performed, identifying a typical batch effect within the replicate experiments, which were hybridised on different days. However the experiment was a balanced designed with each batch containing each treatment group, and was accounted for in the 2 way ANOVA. Lists of significantly changed probe sets (p values less than 0.01 and fold changes of 2 or greater) were generated for each comparison between control and treatment groups (supplementary tables S1-4).

Real-time PCR

One μ g of RNA was reverse transcribed into cDNA using Taqman Reverse Transcriptase. Five μ L of cDNA from each reverse transcriptase reaction was added to 1 μ L of forward primer (20 μ M) and 1 μ L of reverse primers (20 μ M), 5.5 μ L of

nuclease-free water and 12.5 μ L 2 x SYBRgreen PCR Mastermix (Applied Biosystems 4309155). Primers and oligonucleotide probes were designed from cDNA sequences (GenBank) using Primer Express software (Applied Biosystems). Primers were BLAST-searched to verify specificity. Real Time-PCR (RT-PCR) was performed using a Prism 7500 fast real-time PCR machine (Applied Biosystems). The following RT-PCR conditions were used: 40 cycles of 50°C for 2 mins, 95°C for 10 mins, 95°C for 15 secs, and 60°C for 1 min, and for the dissociation curve analysis, 1 cycle at 95°C for 15 sec, 1 cycle at 60°C for 20 sec, and 1 cycle at 95°C for 15 sec. The real time detection cycle was normalised to the 18S signal in each case (Livak and Schmittgen, 2001). Primer sequences for real-time PCR were: Secretogranin 2 Forward 5'-GCAGGGAAGGGAACATCTTG-3', Reverse 5'-AAAGAAGGGAAATTGGGCATTAA-3'; VGF Forward 5'-GCCTCGTCCCCATCTAGTTTC -3' Reverse 5'-TCACACGATTAAATAGAACAGCTTTTC-3'; IGF 2 Forward 5'-TGCAGTTCTCCCCATCATAGG-3' Reverse 5'-ACAAGATGGAGGGCCACACTAG -3'; Galanin Forward 5'-GGAAGTGGAGGAAGGGAGACTAG -3' Reverse 5'-AAACTCCATTATAGTGCGGACGATA -3'; Galanin Receptor 1 (Rat) Forward 5'-TGGACAAAACCTTAGCCACGTAGTTAA -3', Reverse 5'-GCTCATTCGGAATCAAGCATC -3'; Galanin Receptor 2 (Rat) Forward 5'-CCTGTGTCAACCCCATCGTT -3', Reverse 5'-GCGCAGATTTTGCGGAAA -3', Galanin Receptor 3 (Rat) Forward 5'-GCAGTGCCTGTGATCTTTGC -3', Reverse 5'-GCCAACACCAGCCCATTG -3'

Low density survival assay

After 24 hours maturation post PDGF withdrawal, OCs were harvested and plated on to PDL-coated 24-well plates, at 100 cells/well, 20 wells per condition, in 10 μ l of media, and then incubated at 37°C for 5 minutes to settle. Then, 500 μ l of pre-warmed SATO medium, either with or without addition of single growth factors NT3 (5ng/mL), Insulin (5 μ g/mL), LIF (100ng/ml) or recombinant rat galanin (Bachem H-7450, at doses of 0.01-10nM). All plates were then incubated at 37°C for 48 hours. After 48 or 72 hr, calcein AM (4 mM, Invitrogen) and Ethidium homodimer (4mM, Invitrogen) were added to each well and incubated at RT for 30 minutes to identify live/dead cells. Live (green) cells were then counted using an inverted fluorescence microscope.

High density survival assay

After 24 hrs maturation post PDGF withdrawal, OC were harvested and plated at 3 million cells/dish in 10cm PDL-coated plastic dishes, in a total volume of 8 ml of Sato containing NT3 (5ng/ml) and incubated for 48,72 or 96 hrs with or without the addition of LIF (100ng/ml). Calcein AM (4 mM, Invitrogen) and Ethidium homodimer (4mM, Invitrogen) were added to each dish and incubated at RT for 30 minutes to identify live/dead cells. Live (green) cells were then counted using an inverted fluorescence microscope. Ten random fields were counted for each condition.

Oligodendrocyte siRNA

For siRNA transfection experiments, rat OPCs were expanded through two passages into 10 PDL-coated T75 flasks, trypsinised as above and 12 million cells were re-suspended in 410 μ l of rat OC nucleofector solution (Amaxa Biosystems). Then, 100

µl aliquots were transfected (ie. 3 million cells per trial) according to the manufacturer's instructions on program O-17 using an Amaxa Nucleofactor machine, with 1 µl of siRNA solutions directed against galanin, cyclophilin B or non-targeting siRNA solution. After the transfection, 500µl of SATO media was added and the cells transferred to 15cm PDL-coated plates in a total volume of 15 ml of SATO with NT3 (5ng/ml), and incubated with or without LIF (100ng/ml) for 48 or 72 hrs. The siRNA were siCONTROL Cyclophilin B siRNA (D-001136-01-05), siCONTROL Non-targeting siRNA (D-001210-01-05) or rat galanin siGenome smart pool (three siRNA mix), all from Dharmacon RNA technologies, diluted in siRNA buffer to a final siRNA concentration of 20 µM.

In order to establish siRNA efficacy, in 4 independent trials, galanin siRNA reduced galanin mRNA expression in OC 48 hrs post-transfection by an average of 72% compared to non-targeting siRNA transfected cells (range: 57-86%) and by an average of 64% compared to cyclophilin B siRNA transfected cells (range: 56-72%) (data not shown). For these experiments, RNA was extracted from transfected cells using the QIAGEN RNeasy Minikit according to the manufacturer's instructions after washing the plates twice with PBS. The galanin RNA expression was measured using real-time PCR as above. The real time detection cycle was normalized to the 18S signal in each case.

Tumor necrosis factor alpha induced cell death assay

To examine the effect of galanin on cell survival, CG4 cells were seeded at a density of 5,000 cells/well in 8-well chamber slides. Cells were maintained in SATO media for 72 hours in the presence of: (1) no additional factors, (2) recombinant rat galanin (1nM), recombinant rat tumor necrosis factor alpha (100ng/ml R&D systems) and both galanin (1nM) and TNFa (100ng/ml). Ethidium homodimer-1 (4 mM; Invitrogen) and calcein AM (4 mM; Invitrogen) were added to each well followed by visualization under a fluorescent microscope (Carl Zeiss). For cell counts, 5000 cells/field (4 fields per condition) were counted to determine live (calcein AM positive) and dead (ethidium homodimer-1 positive) cells, and expressed as the percentage of live cells per field. Counts were assessed in 3 independent experiments.

Proliferation assay

To label OPCs in S-phase, bromodeoxyuridine (BrdU) (10 µM) was added to the culture medium for 17 hr. Cells were then fixed in 4% PFA for 5 min. After washing, the DNA was denatured in 2 M HCl for 15 min, followed by neutralization in 0.1 M sodium borate for 15 min. Cells were then washed and permeabilized with 20% normal goat serum/0.4% Triton X-100 for 30 min. The cells were incubated with a monoclonal anti-BrdU antibody (1:40; GE Healthcare, Little Chalfont, Buckinghamshire, UK) for 1 h, followed by a goat anti-mouse IgG conjugated with FITC (1:500) for 30 min. The final antibody incubation included Hoechst 33342 (1:2000; Invitrogen) to visualize the nucleus of all cells. For all assays, six wells were counted and assays were performed at least three times with independent cell isolations. All results are shown as percentage of BrdU positive cells +/-SEM. Statistical significance was determined using one-way ANOVAs.

Protein isolation and Western Blots

Optic Nerve-derived rat OCs were grown in 10cm PDL-coated plates until confluence (after passage 1) and then matured overnight in SATO without growth factors. Cells

were treated with LIF for 48 hours (2 doses 24 hrs apart) or left untreated, and cells and media were harvested in protein lysis buffer (1% Triton-X-100, 1% sodium deoxychlorate, 0.1% SDS 150mM NaCl, 10mM Tris pH 7.5, (RIPA), with 1mM iodoacetic acid, 50µg/mL Soybean trypsin inhibitor, 20µg/mL aprotinin, 1mM PMSF, and 1 Complete Protease Inhibitor Mini Tablet, Roche). First we collected 7 ml of supernatant and added 3 mL of protein lysis buffer. Then the cells were washed with cold PBS (GIBCO) twice, lysed in 1mL of protein lysis buffer and harvested using a cell-scalper. Both samples were left 30 mins on ice then centrifuged at 13,000rpm at 4°C for 15 min. The BCA Protein Assay (Pierce) was used to determine protein concentrations. A total of 5µg protein of each cell lysates and 50µg protein from each supernatant lysate was separated on 16% Tricine SDS-PAGE gels (Invitrogen) and transferred to PVDF membranes (Pall Corporation) at 25V for 2 hrs. The blots were blocked in 2% BSA in TRIS-buffered saline containing 0.1% Tween 20 (TBST) for 1 hour, and then incubated overnight at 4°C with primary antibodies including goat polyclonal anti-Secretogranin 2 antibody (M20, Santa Cruz) at 1:200, goat polyclonal anti-VGF antibody (Santa Cruz) 1:200, rabbit anti-IGF 2 antibody (Gropen) 1:1000, anti-Galanin antibody (H80, Santa Cruz) 1:200, goat anti-Gal R2 (K20, Santa Cruz) 1:200. Note that in order to ensure specificity of the H80 anti-galanin antibody for Western blotting we also conducted pre-absorption experiments using recombinant galanin (result not shown). Blots were washed 6 times for 5 min in TBST, all blots were then incubated with secondary antibodies, peroxidase-labeled anti-rabbit or anti-goat IgG at 1:5000 (CST) for 1 hr at RT. Bands were visualized using Amersham Hyperfilm ECL chemiluminescent substrate (Amersham Biosciences). As an additional loading control, all transfer membranes were stained with amido black and photographed.

For the signaling phosphorylation analyses, CG4 cells were grown to confluence in P10 (Nunc) plates in SATO media for 72 hrs with NT3 and PDGF, then differentiated by PDGF withdrawal for 48 hrs. Recombinant galanin was added at 1µg/ml for between 5 and 120 minutes, with an untreated culture used as a “0” minute control. Cells were washed in cold PBS and lysates collected in 100ul RIPA lysis buffer with complete protease and phosphatase inhibitor tablets (Roche) using cell scrapers. Samples of 50 µg of protein lysate in buffer were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes using iBlot (Invitrogen). Proteins were visualized using the Odyssey Infrared Imaging System and quantification tools (LI-COR Biosciences). Primary antibodies were against p-rpS6 (Ser-240/244; Cell Signaling #2215), rpS6 (Cell Signaling #2217), pS-Akt (Ser-473; Cell Signaling #4058), Akt (Cell Signaling #9272), pErk1/2 (p44/42 MAPK, Thr-202/Tyr-204; Cell Signaling #9101), and Erk1/2 (Cell Signaling #4695). Secondary detection antibodies were Donkey anti-Rabbit IgG (IRDye 800CW) and Donkey anti-Mouse IgG (IRDye 680RD).

Immunocytochemistry:

Mature OCs in 24-well plates grown on cover slips were used for immunocytochemistry. The cells were washed in PBS and then fixed by adding 4% PFA for 10 minutes, followed by three washes with PBS. Then, the cells were blocked in 10% Normal Goat Serum/0.3% Triton X in PBS for 2 hr and then stained with primary antibody in the same block: mouse anti-CNPase monoclonal antibody (Chemicon) 1:500; rabbit polyclonal anti-Secretogranin 2 antibody (M20, Santa Cruz)

1:500; or rabbit anti-Galanin antibody (H80, Santa Cruz) 1:200, for 2 hr at RT, followed by 3 washes with PBS (repeated twice for dual epifluorescence staining). Secondary antibodies were then applied to the cells in the same blocking solution, including, anti-mouse Alexafluor 599 (1:400), anti-rabbit fluorescein conjugated secondary antibody (1:400, Jackson) or anti-goat fluorescein conjugated secondary antibody (1:400, Jackson) together with Hoechst 33342 nuclear stain (1:10,000) at RT for 1 hr. After a further 3 PBS washes, the cover slips were mounted on slides using Mowiol (Merck), and photographed using a Zeiss Axioplan 2 Imaging system (Axiovision Software Release 4.4).

Immunohistochemistry:

For fluorescence immunohistochemistry, corpus callosum sections were incubated with blocking solution (2% serum (NGS or NDS), 0.5% BSA, 0.1% Triton-X 100 in 0.1M PBS) for 1h at RT, and then incubated with the appropriate primary antibodies in blocking solution overnight at 4°C: rabbit anti-Galanin (H80, Santa Cruz, 1:200), goat anti-galanin (Abcam, 1:100), mouse anti-CNPase monoclonal antibody (Chemicon, 1:500), rabbit anti-IBA (Wako-Chem, 1:1000), mouse anti-APC (CC-1; Calbiochem, 1:100), rabbit anti NG2 (Millipore, 1:500), mouse anti-GFAP (Millipore, 1:200), rabbit anti-caspase 3 active (Millipore, 1:200). Sections were washed thrice in PBS and then incubated with the appropriate secondary antibodies: goat anti-rabbit IgG fluorescein-conjugated secondary antibody (1:400, Jackson), goat anti-mouse IgG rhodamine-conjugated secondary antibody (1:400, Jackson), donkey anti mouse IgG aminomethyl coumarin acetate conjugated secondary antibody (1:50, Jackson), donkey anti-rabbit IgG rhodamine conjugated secondary antibody (1:200), donkey anti-goat fluorescein-conjugated secondary antibody (1:200) for 2 hours at room temperature. Cell nuclei were labeled with Hoechst 33342 (1:10,000, Invitrogen, Calsbad, CA) in PBS for 10 min at RT and slides were mounted with Mowiol solution (Merck) and coverslipped. Images of stained corpus callosum sections were captured as above. The percentage of IBA1 labeling within the corpus callosum was measured using methods modified from Gresle et al. (2008), where the area of cell nuclei are measured using the threshold and measure functions in ImageJ software (Verison 1.45s)

Statistics

All cell culture assay counts, histological counts and gene expression results were assessed for difference of means using Graphpad Prism 5 for MAC (Version 5). Data distributions were verified as normal. If there were more than two experimental conditions, difference of means was assessed using ANOVA, with Tukey's multiple comparison test. If there were only two conditions, unpaired t-tests were used to compare means. Differences of variance between samples were assessed using Bartlett's test (ANOVA) or F-tests (t-tests).

Results

Cytokine-mediated changes in gene expression within oligodendrocytes

We initially assessed the potential mechanisms of action of three key cytokines known to promote the survival of OCs, namely insulin, NT3, and the interleukin-6 family member, LIF. To assess this, we immuno-purified OC precursor cells from P6 rat optic nerves (Barres et al. 1992), expanded the cells in platelet-derived growth factor (PDGF), at 5ng/ml, differentiated them by PDGF withdrawal for 48 hours, and then exposed the cells to 4 different conditions for 8 hours: basal media only, or basal media supplemented with either LIF (100ng/ml), NT3 (5ng/ml) or insulin (5μg/ml). We harvested RNA from three independent replicate experiments (two independent replicates for the basal condition), and utilized gene expression microarrays (Affymetrix) to compare differential gene expression profiles between conditions. Lists of significantly differentially expressed (DE) genes (p values less than 0.01 and fold changes of 2 fold or greater, ANOVA) were generated for each comparison between control and treatment groups (Tables 1-3, with 3-fold or greater shown for LIF versus control). There was little concordance between genes induced by LIF, insulin or neurotrophin-3. Some 95 probe sets were DE between control and LIF-treated cells, 15 between control and NT3-treated cells and 35 between control and insulin-treated cells. Of the 95 genes DE in the LIF condition, 83 were increased and 12 decreased. Eighty-six of the DE genes were uniquely regulated in the LIF condition. Nine probe sets changed in multiple growth factor conditions (see Tables 1-3), namely LIF and Insulin (n=2, Probeset ID 1376891 and Ate1); LIF and NT3 (n=6, Probeset ID 1375268, Smg5, Dcun1d1, Mobp, Gdpd1 and Appbp2) or all three (n=1, Probeset ID 1380200).

LIF regulates genes encoding secretory proteins

The list of 95 differentially expressed probe sets following LIF treatment was then mined for gene ontology (GO) terms in order to identify major biological themes using GOMiner (Zeeberg et al. 2003). The enrichment analysis from GOMiner was then analysed using Vennmaster (Kestler et al. 2008). Overrepresented GO categories for “Cellular Component” were filtered with a p-value of less than 0.02 and category size bracket of 70-400. We identified 14 overlapping categories containing a total of 17 genes. Notable significantly over-represented categories were “cytoplasmic vesicles”, “secretory granules” and “extracellular space”. In particular, we noted from within these categories that LIF induced the expression of genes encoding the secreted peptides galanin, VGF, calcitonin-related peptide (CRP), insulin-like growth factor 2 (IGF2) and the secretory granule protein secretogranin II (Figure 1a).

We performed real-time PCR validation of these five upregulated genes using 3 independent OC culture replicates, comparing exposure to LIF for 8 hrs with exposure to insulin for 8 hrs, and confirmed highly significant LIF-induced gene expression increases for all of them (Fig 1b). We also compared RNA expression of the same 5 candidates in independent duplicate OC cultures treated with either LIF in combination with insulin or insulin alone for 24 hrs, and again demonstrated significant LIF-induced gene induction for all 5 RNA species (data not shown).

LIF-induced secretion of the neuropeptide galanin in oligodendrocytes in vitro

We next sought to identify whether LIF-mediated induction of these five mRNA transcripts in OC also resulted in upregulation of their respective encoded proteins. In order to maximise cellular survival, we chose to conduct these experiments in OC cultures in the presence of insulin. We exposed high-density OC cultures to either LIF (100ng/ml) or “no added factor” for 48 hrs and harvested the culture medium. We concentrated the OC-conditioned medium by size-selective centrifugation and performed Western blots to assess for the presence and LIF-induced regulation of galanin, CRP, VGF, IGF-2 and secretogranin-2. In duplicate experiments, an anti-galanin antibody detected a strong LIF-induced increase in a band at approximately 14 kD (Fig 2a), corresponding to the reported size of secreted preprogalanin (Lang et al. 2007; Yu et al. 2007). We also exposed high-density cultures of the oligodendroglial cell line, CG4, to either LIF (100ng/ml) or “no additional factor” for 48 hrs (all in the presence of insulin) and once again identified a 14 kD band by Western blot analysis that was significantly induced by LIF. In order to assess cellular specificity of the response, we also collected conditioned medium from duplicate primary rat astrocyte cultures exposed to either LIF or “no added factor” for 48 hrs and detected no significant LIF-induced increase of the same 14 kD band (Fig 2a). In these experiments, we could also detect expression of two isoforms of secretogranin-2 and demonstrated possible regulation of the 30kD isoform of secretogranin-2 by LIF exposure (data not shown), consistent with prior reports showing regulation of a 30kD isoform in melanocytes exhibiting a secretory phenotype when compared to non-secretory melanotrope cells (Peinado et al. 2006). Using immunohistochemistry (IHC), we were also able to visualize LIF-mediated induction of galanin and secretogranin-2 in OC *in vitro* in a topographic pattern consistent with their location in cytoplasmic granules (Fig.3, panels a-d).

We also detected IGF2 in the conditioned OC medium, but this was not regulated by LIF (Fig. 2b). We found no evidence for LIF-mediated secretion of CRP or VGF proteins using Western blots (data not shown).

LIF-induced expression of the neuropeptide galanin in oligodendrocytes *in vivo*

We next sought evidence for LIF-induced galanin secretion in OC *in vivo*. We utilized the cuprizone model (Matsushima and Morell 2001), which produces a toxic demyelinating lesion in the corpus callosum. Of relevance to our focus, cuprizone is known to induce the production of both LIF and CNTF in the demyelinating corpus callosum (Emery et al. 2006). We exposed wild type C57Bl6 mice (n=6), LIF knockout mice (n=5), CNTF knockout mice (n=4) and LIF/CNTF double knockout mice (n=2) to 18 days of cuprizone ingestion, an early demyelinating time-point. We performed dual fluorescent staining for galanin and the OC marker CNPase in the caudal corpus callosum of these mice and compared staining patterns to those obtained in control mice of the same genotypes without cuprizone challenge (n=2/genotype). We assessed the central corpus callosum in three coronal sections per mouse. In control mice, we did not detect any co-localisation of galanin and CNPase staining (data not shown). In cuprizone-exposed wild-type mice, 76% (SE +/-9%) of OC expressed galanin (Fig 3e), whereas in LIF knockout mice, only 18% (SE +/-10%) of OC expressed galanin (Fig 3f, $p=0.008$ compared to wild-type mice). In CNTF/LIF double knockout mice, galanin expression was detected in 9% (SE +/-5%) of CNPase positive cells ($p=0.04$ compared to wild-type mice). In contrast, 66% (SE +/-17%) of CNPase positive cells expressed galanin in CNTF knockout mice, not significantly different from wild-type mice ($p=0.47$).

To independently confirm cuprizone-induced OC expression of galanin, we assessed sections from transgenic mice expressing the fluorophore dsRED under the control of the OC-specific Proteolipid protein promoter (PLP-dsRED, Hirrlinger et al, 2005). Six transgenic animals were exposed to cuprizone for three weeks, killed and processed for galanin immunohistochemistry. As a positive staining control, we identified galanin-staining in nerve fibres and cells of the paraventricular thalamic nucleus (Fig 3g), and we demonstrated that PLPdsRed positive OC in this area, which is not affected by toxic cuprizone demyelination, did not express galanin (Fig. 3g). Oligodendrocytic expression of galanin was restricted to the caudal corpus callosum, where 75% (SE \pm 11%) of PLPdsRed positive cells expressed the neuropeptide (Fig 3h) and high power light microscopy revealed an intracellular, granular pattern of galanin staining, consistent with galanin presence in OC secretory vesicles (Fig 3i). It was also observed that galanin co-labeled with a small number of microglia/macrophages within the demyelinated CC (Fig S1 A), but not GFAP labeled astrocytes (Fig S1 A) or NG2 labeled OC progenitor cells (Fig S1 B). Very few activated caspase 3 (apoptotic) labeled OCs were observed in the CC after 3 weeks of cuprizone treatment, and none of these were found to co-label with galanin (Fig S1 C).

Galanin promotes the survival of GalR2 expressing oligodendrocytes

Having established that OCs regulate galanin expression during central demyelination, we next assessed whether, as a secreted protein, galanin might also exert autocrine/paracrine effects. We therefore assessed the survival of post-mitotic rat OC in vitro 48hr after addition of recombinant galanin at 0.01nM, 0.1nM, 1nM, 10nM, 100nM and 1 μ M, compared to a basal media (Sato only) control condition. Galanin concentrations at or above 1nM significantly increased OC survival. The survival effect was maximal at 1nM galanin, which increased OC survival by 50%, $p=0.0008$ (ANOVA). An independent replicate experiment confirmed this result, with the 1nM galanin condition increasing OC survival 46% above baseline at 48 hrs ($p=0.005$). In a third independent experiment, we compared OC survival induced by galanin with that induced by known OC survival factors, namely LIF, NT3 and insulin after 48 hours of exposure to each factor. The survival of OC cultured with galanin was 87% above the Sato basal media condition ($p=0.004$), and was not significantly different from the survival effect induced by LIF or NT3 (Fig 4a), although less than that produced by insulin. The survival effect of 1 nM galanin on OC was still apparent at 72 hrs in culture compared to the Sato basal media condition ($p=0.016$, Fig 4b).

We were also able to demonstrate that exogenous galanin (1nM) can promote the survival of OCs derived from CG4 cells at 72 hours of exposure to TNF α (100ng/ml) (figure S1 D; Galanin and TNF α condition ($40.6 \pm 1.4\%$) vs TNF α only ($17.8 \pm 5.1\%$)).

We next assessed whether galanin promoted proliferation of actively dividing OPC. We did not detect a proliferative effect of galanin compared to the Sato media negative control condition, whereas the known OPC mitogen platelet-derived growth factor (PDGF, Raff et al, 1988) at a known maximal concentration of 5ng/ml, induced division of an estimated 45% of all OPC within 24 hrs. Galanin combined with half-maximal PDGF (2.5ng/ml) also did not promote OPC division above the level seen with half-maximal PDGF alone (Fig. 4c).

In neurons, galanin is known to signal via three different galanin receptors (GalR1, 2 and 3), all of which are G-protein-coupled receptors (reviewed by Hokfelt, 2005). Having demonstrated a significant survival effect of galanin upon OC, we next sought evidence of expression of any of the three known galanin receptors in these cells. The mRNA levels of GalR2 was 100 fold greater than GalR3 and 1000 fold greater than GalR1 in OC grown in insulin for 48 hrs (Fig. 4d).

Galanin signaling in OC phosphorylates ERK1/2

In order to investigate signaling pathways potentially activated by galanin in OC, we performed a time-course experiment to assess phosphorylation of ERK1/2, AKT and S6 in CG4 cells in triplicate. In each experiment, ERK1/2 was rapidly phosphorylated after administration of recombinant galanin (1 µg/ml), whereas there was no consistent evidence for AKT or S6 phosphorylation (Fig 4e).

LIF-induced oligodendrocyte survival is dependent on galanin

Given our demonstration of LIF-mediated galanin secretion and of galanin-induced survival of OC, we hypothesized that LIF-induced survival of OC could be partially or completely dependent on galanin secretion. To test this hypothesis we contemporaneously assessed the survival of post-mitotic OC differentiated from OPC isolated from wild type or galanin knockout (Gal-KO) mice. In independent duplicate experiments, the cells were plated at high density in Sato media with or without LIF (100ng/ml). High density culture was used because we hypothesized that LIF-mediated galanin secretion into the culture medium could then act as an OC survival factor, an effect that could not be observed with low-density or clonally plated cells in standard survival assays. OC survival in Sato media (basal condition) was no different between genotypes at 48, 72 or 96 hours subsequent to plating of the OPC. As expected, LIF induced a pro-survival effect in wild-type OC cultures, but, importantly, LIF did not potentiate the survival of Gal-KO OC cultures at any time-point (Figure 5). In contrast, we confirmed that galanin itself retained its survival effect in OC derived from GAL-KO mice. At 48 hrs, 1nM galanin increased survival of these OC by 45% above SATO alone, (SD 32-56%, $p < 0.01$, $n = 8$ plates of 16 wells/group) and the effect was maintained at 72 hrs (mean 77% survival increase, SD 53-99%, $p < 0.01$, $n = 8$ plates of 16 wells/group, 100 cells/well), thus demonstrating that the lack of LIF-mediated survival in GAL-KO OC is not secondary to altered galanin-signaling in the KO cells.

Secondly, we established small inhibitory RNA mediated galanin knockdown in rat OC in order to independently verify the above survival experiment. We used rat OC subjected to either si-RNA mediated knockdown of galanin, or non-targeting siRNA transfection as a control, in high-density cultures. We conducted independent triplicate experiments. We plated either non-targeting siRNA or galanin siRNA transfected OC in basal media plus insulin, and compared cell survival with or without addition of LIF (100ng/ml). In OC transfected with non-targeting siRNA, LIF addition produced an average 60% survival increase at 48 hrs (Individual results: +80%, +25% and +75%, compared to no LIF addition, $p = 0.04$, T-test), and this was abolished in galanin siRNA transfected cells, with an average 0% survival (+20%, -20%, +/-0%, $P = \text{NS}$) in LIF-treated versus untreated OC cultures).

Galanin KO mice exhibit normal myelination but develop more severe callosal demyelination during cuprizone exposure

Since we have shown that galanin is expressed in callosal OC during cuprizone-induced demyelination (see above), and that it is a survival factor for OC *in vitro*, we next assessed the potential significance of galanin expression during toxic demyelination *in vivo*. We exposed wild type and (Gal-KO) mice to cuprizone for 4 weeks and assessed the density of myelinated axons in the severely demyelinated caudal corpus callosum. We performed experiments with a total of 12 mice/group. In the caudal mid-line corpus callosum (Fig 6a,b,c), Gal-KO mice had significantly fewer myelinated axons (mean 56, sd +/- 7 myelinated axons/field) than wild-type mice (mean 125, sd +/- 15 myelinated axons/field), $p=0.0004$ (Fig.5d). Of note, the density of myelinated axons in the caudal mid-line corpus callosum was not different in control wild-type (mean 2020, range 1700-2090 myelinated axons/field) and Gal-KO mice (mean 2100, range 1650-2380 myelinated axons/field; $n=6$) in the absence of cuprizone challenge.

We next assessed OC density in mice ($n=6$ /group). In the caudal, cuprizone affected corpus callosum (identified by the presence of macrophage infiltrates), Gal-KO mice showed a 2.4-fold reduction in CC1-positive OC density, with a mean OC density of $46.4/\text{mm}^2$ (SE $8.2/\text{mm}^2$) in GAL-KO mice ($n=6$) and $125/\text{mm}^2$ (SE $39/\text{mm}^2$) in wild-type mice ($n=6$), $P=0.05$.

In the rostral CC of cuprizone treated mice, mean OC density in Gal-KO mice ($n=6$) was $433/\text{mm}^2$ (SE $71/\text{mm}^2$), and $461/\text{mm}^2$ (SE $60/\text{mm}^2$) in WT mice ($n=6$), $P=0.77$.

In the mid-sagittal section, the maximum width of the cuprizone-affected caudal corpus callosum, an indicator of callosal swelling, was no different between Gal-KO mice and healthy wild-type mice (data not shown), indicating that axon density changes were not a function of altered inflammation-associated edema in the different genotypes. The percentage of callosal area labeled with IBA1 was not different between WT ($25.1 \pm 6.8\%$) and Gal-KO ($26.2 \pm 5.0\%$) mice after 3 weeks of cuprizone treatment.

Discussion

In this study, we report a number of findings of significance with respect to OC biology and survival. Firstly, three cytokines known to be important for OC survival induce the transcription of a range of genes. However these down-stream cytokine-dependent gene expression profiles are disparate, suggesting that there are probably no common survival pathways that underlie their biological activity. Secondly, we report that LIF-treated post-mitotic OC secrete a number of neuropeptides, in particular galanin, at the mRNA and protein level. Our experiments confirm LIF-induced galanin protein synthesis and secretion in primary rat OC, but not in astrocytes. *In vitro*, galanin itself was shown to be a survival factor for OC, and its induction was required for LIF-mediated OC survival in high-density culture. *In vivo*, galanin expression was induced in OCs in response to cuprizone-induced demyelination and also reduced the extent of myelin loss in this disease model. We also demonstrated that galanin induction in corpus callosum OC during cuprizone-induced demyelination is largely dependent on endogenous LIF, concordant with our *in vitro* results.

We have previously shown that LIF mRNA is markedly upregulated in the corpus callosum of mice challenged with cuprizone. The LIF knockout mice exhibit increased demyelination compared to wild-type littermates (Emery et al. 2006) whereas systemic administration of recombinant murine LIF reduces demyelination in comparison to placebo-treated mice (Marriott et al. 2008). Based on the observations from our current study, we postulate that LIF mediates myelin protection during cuprizone toxicity through induction of galanin secretion in OCs. It is also possible that galanin could have targeted commissural axons within the corpus callosum to make them more receptive to remyelination or to resist demyelination after cuprizone challenge. The distinction between these possibilities or the determination that both are relevant will require the use of conditional knockout strategies in which the relevant Gal receptors are selectively ablated from either neurons or OCs

In hippocampal slice cultures, recombinant galanin rapidly phosphorylates both ERK1/2 and AKT (Elliott-Hunt et al. 2007). In OC, we have demonstrated that galanin rapidly induces ERK1/2 phosphorylation, but not AKT or rpS6 phosphorylation, suggesting some differences in signaling mechanisms between neurons and OC. This provided direct evidence of galanin signaling in OC. The three galanin receptors are differentially expressed in OC, with the relative expression profiles suggesting that GalR2 is likely to be the relevant receptor. However, Zhang and coworkers reported a two-fold upregulation of GalR1 mRNA expression in the cuprizone-affected corpus callosum at 6 weeks compared to tissue obtained from healthy controls (Zhang et al. 2012). Ultimately, galanin receptor mRNA change may or may not indicate the precise signaling mechanism, and thus, future work should include the conditional deletion of one or more of the receptor species in OC, or the assessment of OC responses to specific galanin agonists to further delineate potential galanin receptor specificity and inform therapeutic studies.

Collectively, our results demonstrate induction of peptide secretion as a novel and biologically significant cellular function in post-mitotic OC. We show that OCs upregulate expression of the neuropeptide galanin, and that this response occurs *in vivo* in the context of demyelinating injury. We also demonstrate that, *in vitro*, galanin secretion by OC probably sub serves an autocrine or paracrine survival loop and that *in vivo* OC-derived galanin directly reduces the extent of demyelination in the cuprizone injury model. We further show that galanin upregulation in OC in this model is downstream of endogenous LIF regulation, a biological response that was previously described in neurons. We conclude that galanin signaling could be an important determinant of outcome in central demyelinating disease and, as such, could represent an important therapeutic target for multiple sclerosis.

References

- Alonzi T, Middleton G, Wyatt S, Buchman V, Betz UA, Muller W, Musiani P, Poli V, Davies AM. 2001. Role of STAT3 and PI 3-kinase/Akt in mediating the survival actions of cytokines on sensory neurons. *Molecular and Cellular Neurosciences* 18:270-82.
- Barres BA, Hart IK, Coles HS, Burne JF, Voyvodic JT, Richardson WD, Raff MC. 1992. Cell death in the oligodendrocyte lineage. *J Neurobiol* 23:1221-30.
- Barres BA, Schmid R, Sendtner M, Raff MC. 1993. Multiple extracellular signals are required for long-term oligodendrocyte survival. *Development* 118:283-95.
- Binder MD, Cate HS, Prieto AL, Kemper D, Butzkueven H, Gresle MM, Cipriani T, Jokubaitis VG, Carmeliet P, Kilpatrick TJ. 2008. Gas6 deficiency increases oligodendrocyte loss and microglial activation in response to cuprizone-induced demyelination. *Journal of Neuroscience* 28:5195-206.
- Boulton TG, Stahl N, Yancopoulos GD. 1994. Ciliary neurotrophic factor/leukemia inhibitory factor/interleukin 6/oncostatin M family of cytokines induces tyrosine phosphorylation of a common set of proteins overlapping those induced by other cytokines and growth factors. *Journal of Biological Chemistry* 269:11648-55.
- Bugga L, Gadiant RA, Kwan K, Stewart CL, Patterson PH. 1998. Analysis of neuronal and glial phenotypes in brains of mice deficient in leukemia inhibitory factor. *Journal of Neurobiology* 36:509-24.
- Cahoy JD, Emery B, Kaushal A, Foo LC, Zamanian JL, Christopherson KS, Xing Y, Lubischer JL, Krieg PA, Krupenko SA and others. 2008. A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. *Journal of Neuroscience* 28:264-78.
- Casaccia-Bonnel P. 2000. Cell death in the oligodendrocyte lineage: A molecular perspective of life/death decisions and the development of disease. *Glia* 29:124-135.
- Elliott-Hunt CR, Pope RJ, Vanderplank P, Wynick D. 2007. Activation of the galanin receptor 2 (GalR2) protects the hippocampus from neuronal damage. *J Neurochem* 100:780-9.
- Emery B, Cate HS, Marriott M, Merson T, Binder MD, Snell C, Soo PY, Murray S, Croker B, Zhang JG and others. 2006. Suppressor of cytokine signaling 3 limits protection of leukemia inhibitory factor receptor signaling against central demyelination. *Proceedings of the National Academy of Sciences of the United States of America* 103:7859-64.
- Ernst M, Novak U, Nicholson SE, Layton JE, Dunn AR. 1999. The carboxyl-terminal domains of gp130-related cytokine receptors are necessary for suppressing embryonic stem cell differentiation. Involvement of STAT3. *Journal of Biological Chemistry* 274:9729-37.
- Gresle MM, Shaw G, Jarrott B, Alexandrou EN, Friedhuber A, Kilpatrick TJ, Butzkueven H. 2008. Validation of a novel biomarker for acute axonal injury in experimental autoimmune encephalomyelitis. *J Neurosci Res* 86:3548-55.

- Hirrlinger PG, Scheller A, Braun C, Quintela-Schneider M, Fuss B, Hirrlinger J, Kirchhoff F. 2005. Expression of reef coral fluorescent proteins in the central nervous system of transgenic mice. *Molecular and Cellular Neurosciences* 30:291-303.
- Hokfelt T. 2005. Galanin and its receptors: introduction to the Third International Symposium, San Diego, California, USA, 21-22 October 2004. *Neuropeptides* 39:125-42.
- Kahn MA, De Vellis J. 1994. Regulation of an oligodendrocyte progenitor cell line by the interleukin-6 family of cytokines. *Glia* 12:87-98.
- Kestler HA, Muller A, Kraus JM, Buchholz M, Gress TM, Liu H, Kane DW, Zeeberg BR, Weinstein JN. 2008. VennMaster: area-proportional Euler diagrams for functional GO analysis of microarrays. *BMC Bioinformatics* 9:67.
- Lang R, Gundlach AL, Kofler B. 2007. The galanin peptide family: receptor pharmacology, pleiotropic biological actions, and implications in health and disease. *Pharmacology and Therapeutics* 115:177-207.
- Marriott MP, Emery B, Cate HS, Binder MD, Kemper D, Wu Q, Kolbe S, Gordon IR, Wang H, Egan G and others. 2008. Leukemia inhibitory factor signaling modulates both central nervous system demyelination and myelin repair. *Glia* 56:686-98.
- Masu Y, Wolf E, Holtmann B, Sendtner M, Brem G, Thoenen H. 1993. Disruption of the CNTF gene results in motor neuron degeneration. *Nature* 365:27-32.
- Matsushima GK, Morell P. 2001. The neurotoxicant, cuprizone, as a model to study demyelination and remyelination in the central nervous system. *Brain Pathology* 11:107-16.
- McCarthy KD, de Vellis J. 1980. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *Journal of Cell Biology* 85:890-902.
- Peinado JR, Vazquez-Martinez R, Cruz-Garcia D, Ruiz-Navarro A, Anouar Y, Tonon MC, Vaudry H, Gracia-Navarro F, Castano JP, Malagon MM. 2006. Differential expression and processing of chromogranin A and secretogranin II in relation to the secretory status of endocrine cells. *Endocrinology* 147:1408-18.
- Raff MC, Lillien LE, Richardson WD, Burne JF, Noble MD. 1988. Platelet-derived growth factor from astrocytes drives the clock that times oligodendrocyte development in culture. *Nature* 333:562-5.
- Sadowski HB, Shuai K, Darnell JE, Jr., Gilman MZ. 1993. A common nuclear signal transduction pathway activated by growth factor and cytokine receptors. *Science* 261:1739-44.
- Tomida M, Heike T, Yokota T. 1999. Cytoplasmic domains of the leukemia inhibitory factor receptor required for STAT3 activation, differentiation, and growth arrest of myeloid leukemic cells. *Blood* 93:1934-41.
- Wraith DC, Pope R, Butzkueven H, Holder H, Vanderplank P, Lowrey P, Day MJ, Gundlach AL, Kilpatrick TJ, Scolding N and others. 2009. A role for galanin in human and experimental inflammatory demyelination. *Proceedings of the National Academy of Sciences of the United States of America* 106:15466-71.
- Wynick D, Small CJ, Bacon A, Holmes FE, Normal M, Ormandy CJ, Kilic E, Kerr NCH, Ghatei M, Talamantes F and others. 1998. Galanin Regulates

- prolactin release and lactotroph proliferation. *Proceedings of the National Academy of Sciences of the United States of America* 95:12671-12676.
- Yu JY, Pon CH, Ku HC, Wang CT, Kao YH. 2007. A preprogalanin cDNA from the turtle pituitary and regulation of its gene expression. *Am J Physiol Regul Integr Comp Physiol* 292:R1649-56.
- Zeeberg BR, Feng W, Wang G, Wang MD, Fojo AT, Sunshine M, Narasimhan S, Kane DW, Reinhold WC, Lababidi S and others. 2003. GoMiner: a resource for biological interpretation of genomic and proteomic data. *Genome Biol* 4:R28.
- Zhang L, Yu W, Schroedter I, Kong J, Vrontakis M. 2012. Galanin transgenic mice with elevated circulating galanin levels alleviate demyelination in a cuprizone-induced MS mouse model. *PLoS One* 7:e33901.

Figure Legends:

Figure1: LIF-induced gene expression changes in oligodendrocytes in vitro

Figure 1A: Gene array expression levels of selected candidate genes classified by the cellular process code “secretion” that were significantly up-regulated in LIF conditions (n=3) compared to no growth factor control (n=2), insulin (n=3) or NT3 (n=3). The Y-axis in each panel is the relative expression (log base 2) for each condition, averaged by least squared mean.

Expression levels of each of the 6 genes were a least 2-fold greater in the LIF condition than the other conditions, $*=p<0.01$, for specific p-values see Table 1 (error bars: SEM).

Figure 1B: Real-time PCR validation: relative oligodendrocyte RNA expression levels of the same six genes after LIF exposure for 8hrs, compared to insulin exposure for 8 hrs (n=3 independent replicates). Lowest expression level in insulin condition was set to 1, and results illustrate the mean (SD) expression change, $p<0.05$ for each gene expression comparison between LIF and Insulin conditions.

Figure 2: LIF-induced protein changes in oligodendrocytes and conditioned medium

Figure 2a: Concentrated media of rat oligodendrocyte (OC, upper panel) and CG4 cell cultures (middle panel), cultured for 48 hrs, were subject to Western blotting using anti-galanin antibodies. Cultures exposed to LIF at 100ng/ml showed marked upregulation of a 14kD band consistent with secretion of preprogalanin in comparison to cells exposed only to insulin (Nil). Rat astrocytes (lower panel) did not exhibit the same LIF-induced response (100ng/ml, 48 hrs). Independent replicate experiments are shown in each blot, and major protein bands visualized by Amido Black stains of the same membranes are shown as loading controls. Figure 2b: Western blot of concentrated media of OC cultures exposed to either LIF or insulin showed evidence of a band at 7kD, consistent with IGF2 secretion. Unlike preprogalanin, IGF2 levels were not increased by LIF at the protein level.

Figure3: Galanin-containing secretory granules in oligodendrocytes are induced by LIF in vitro and in vivo

CNPase positive oligodendrocytes (OC) (red) in culture containing insulin (panel a) showed no galanin expression (green). After exposure to LIF (100ng/ml, 48 hrs), CNPase positive OC (panel b) showed punctate cytoplasmic galanin staining (arrow). CNPase positive OC (c, red) showed some granular cytoplasmic staining for secretogranin-2 (green), but this was markedly upregulated after 48hrs of LIF exposure (panel d, arrow). CNPase (red) positive OC were visualized in linear arrays in the corpus callosum (cc, panels e,f). After in vivo exposure to cuprizone for 18 days, 75% of OC in the corpus callosum of wild-type mice exhibited cytoplasmic galanin immunoreactivity (green, panel e, arrows), whereas OC in the cc of LIF knockout mice exhibit no galanin positivity (panel f). Images in panels g-i are from PLP-dsRED mice expressing the dsRED fluorophore, stained with anti-galanin antibody (green). After 21 days of systemic cuprizone exposure, in the paraventricular thalamic nucleus (panel g), galanin-positive neuronal cell bodies and fibres were seen next to galanin-negative OC (red). In contrast, in the demyelinating cc around 75% of PLP-ds red positive OC expressed cytoplasmic galanin (panel h, arrows). Occasional dsRED negative cells also expressed galanin (arrowhead). Panel i: high power image from the cc illustrating granular cytoplasmic galanin staining in a dsRED positive cell. Scale bars for panels a-f,i=10µm; for panels g and h=50 µm.

Figure 4: Galanin is a survival factor for oligodendrocytes

Figure 4a and 4b: Oligodendrocyte (OC) survival 48 or 72 hrs after addition of either recombinant rat galanin (3.2ng/ml=1nM), LIF (100ng/ml), NT3 (5ng/ml) or insulin (5μg/ml), compared to basal media only. Post-mitotic OC were plated at 100 cells/well (20 wells per condition), and the data are presented as live cell counts (mean +/-SD). Each growth factor condition significantly increased survival above basal (*p<0.05, ***p<0.001, ANOVA). Figure 4c: To assess any proliferative effect of galanin, oligodendrocyte progenitor cells (OPCs) were exposed to galanin (1nM), PDGF (5ng/ml), PDGF (2.5 ng/ml) and the combination of PDGF (2.5ng/ml) and galanin (1nM). Maximal-dose PDGF (5ng/ml) induced around 40% BRDU-positivity, whereas galanin induced none. The combination of half-maximal dose PDGF and galanin did not promote OPC proliferation above that induced by half-maximal dose PDGF alone (*** denotes significant difference to basal proliferation rate, p<0.001, ANOVA, data shown are mean % +/-SD). Figure 4d: Relative mRNA expression of the three known galanin receptor species in post-mitotic OC assessed by real-time PCR. GalR2 mRNA is around 900 fold more abundantly expressed than the other receptor species (mean of three separate experiments shown, lowest GalR1 expression was standardized to 1, error bars = SD, *** denotes significant difference compared to GalR1 expression, p<0.001, ANOVA). Figure 4e: Time course of phosphorylation of key signaling intermediaries after galanin (1μg/ml) exposure in vitro. ERK 1/2 is rapidly phosphorylated, whereas AKT and S6 phosphorylation levels are unaltered. Representative images of duplicate experiments shown.

Figure 5: LIF-mediated oligodendrocyte survival in vitro is galanin-dependent
Mouse oligodendrocyte progenitor cells from wild-type (WT) and galanin knockout (Gal KO) mice were expanded in vitro and then plated under differentiating conditions at high density in Sato (Nil) or Sato plus LIF (100ng/ml). Live cells were counted at 48, 72 and 96 hrs under bright field microscopy (average of 10 fields in each of two plates/condition). Results were combined from independent duplicate experiments, and survival is expressed as mean (+/-SD) live cell count/field. Addition of LIF to WT oligodendrocyte (OC) cultures increased survival at 48, 72 and 96 hrs. Addition of LIF to Gal KO OC cultures did not produce a survival effect at any time-point. (***p<0.001; *p<0.05, ANOVA).

Figure 6: Galanin knockout mice exhibit more severe cuprizone demyelination.
Galanin knockout (KO) mice (n=11) and wildtype mice (n=11) were fed cuprizone 0.2% in milled chow for 4 weeks. Myelinated axon density was quantitated in the severely demyelinated caudal portion of the corpus callosum (Fig 6a, black square) in two fields/mouse from methylene-blue stained thin sections (6b,c). Panel 6b illustrates severe loss of myelinated axons in a Galanin KO mouse, panel 6c shows less severe loss from a wild-type mouse, arrowheads indicate myelinated axons, scale bars =40 μm, unit area=17400μm². Galanin KO mice had less residual myelinated axons than wild-type mice (Fig 6d, average myelinated axon densities from individual mice shown in scatterplot, lines indicate means, ***p<0.001, t-test).

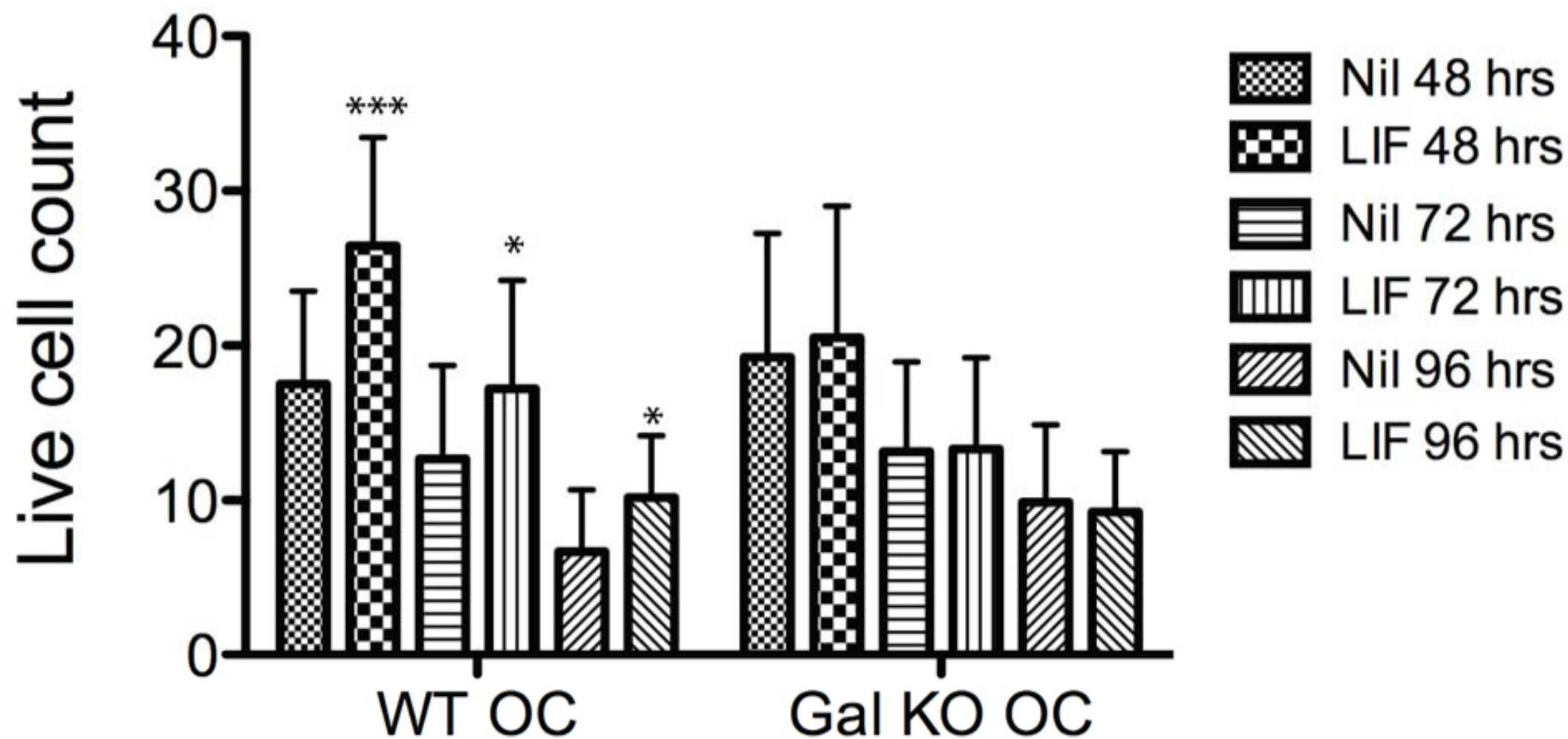
Table 1: Probe sets with differential expression between control and LIF treatment (p<0.01 and fold change >2). Positive fold changes indicate increased expression in control samples compared to treated samples. Probeset ID refers to Affymetrix probe sets.

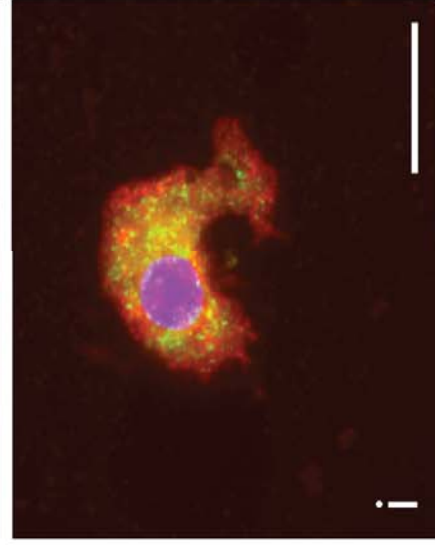
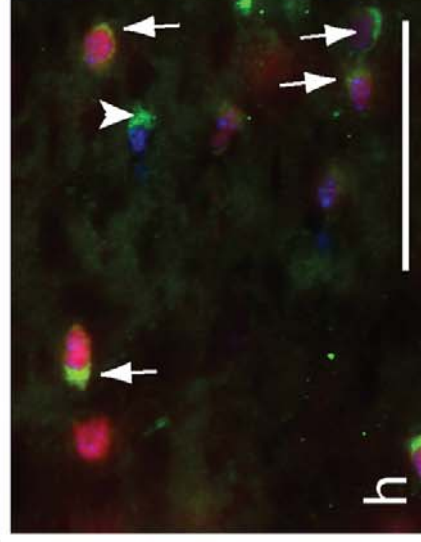
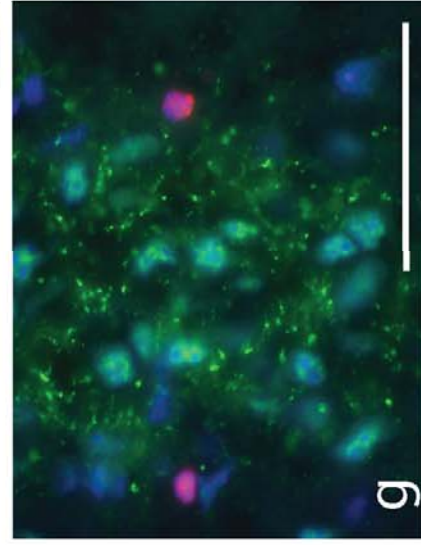
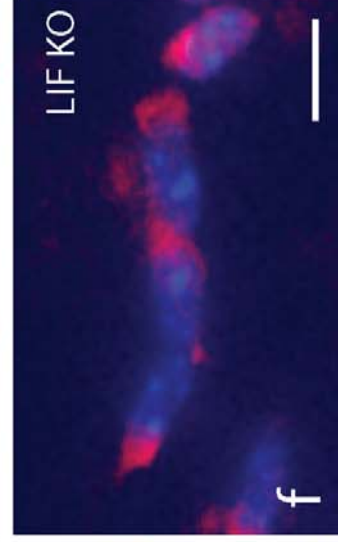
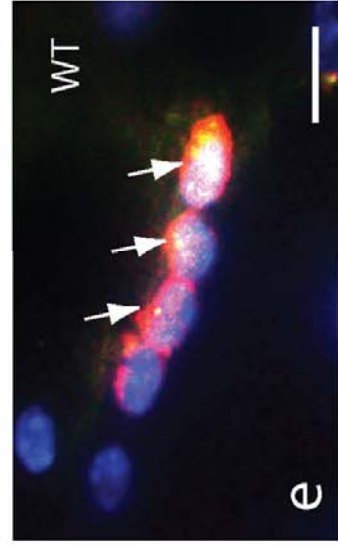
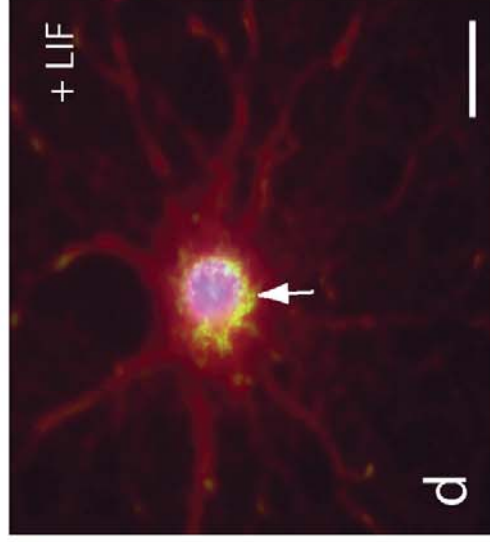
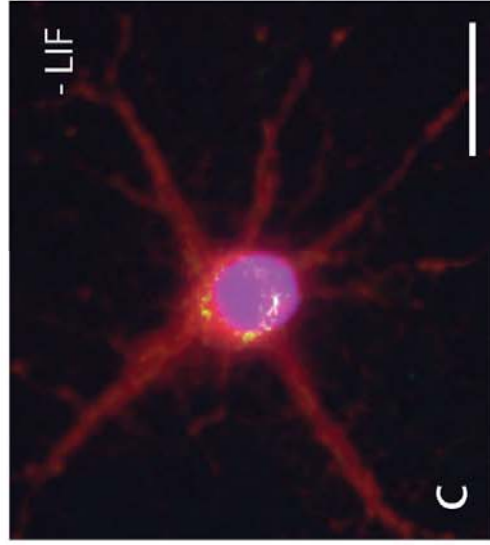
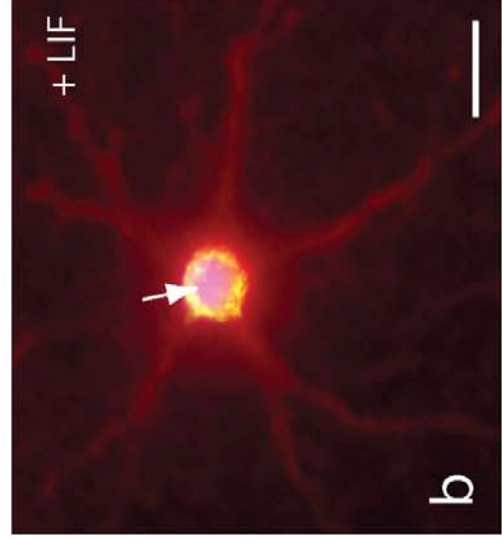
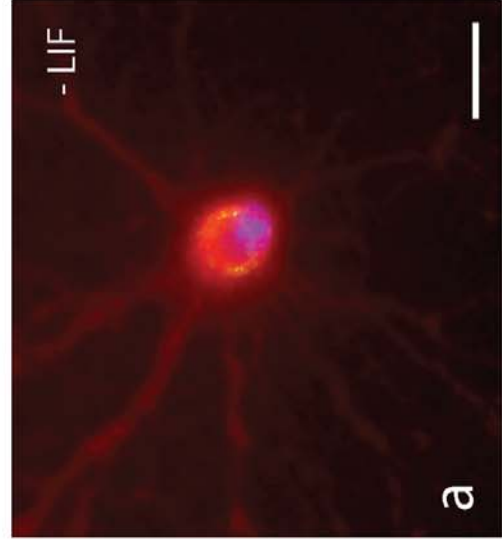
Table 2: Probe sets with differential expression between control and NT3 treatment ($p < 0.01$ and fold change > 2). Positive fold changes indicate increased expression in control samples compared to treated samples. Probeset ID refers to Affymetrix probe sets.

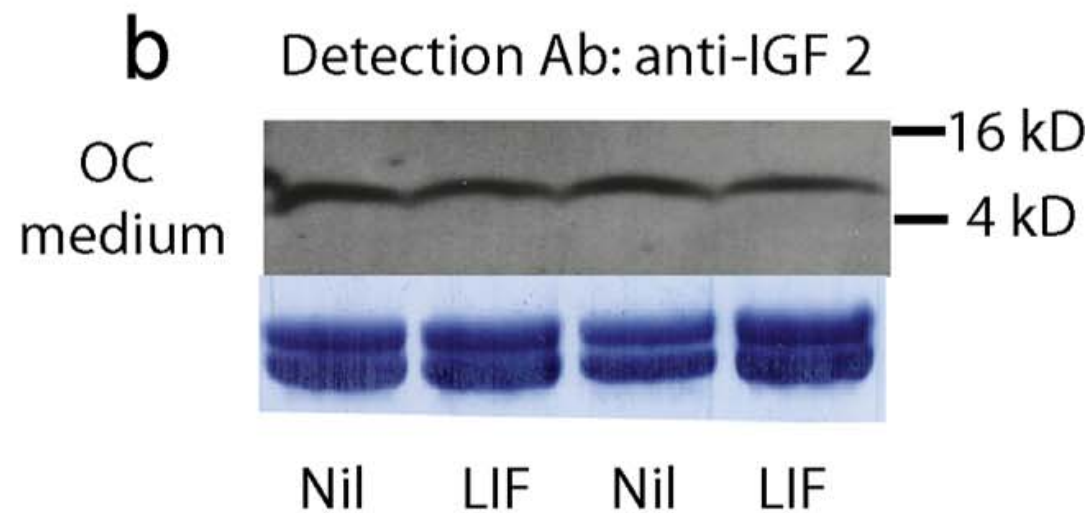
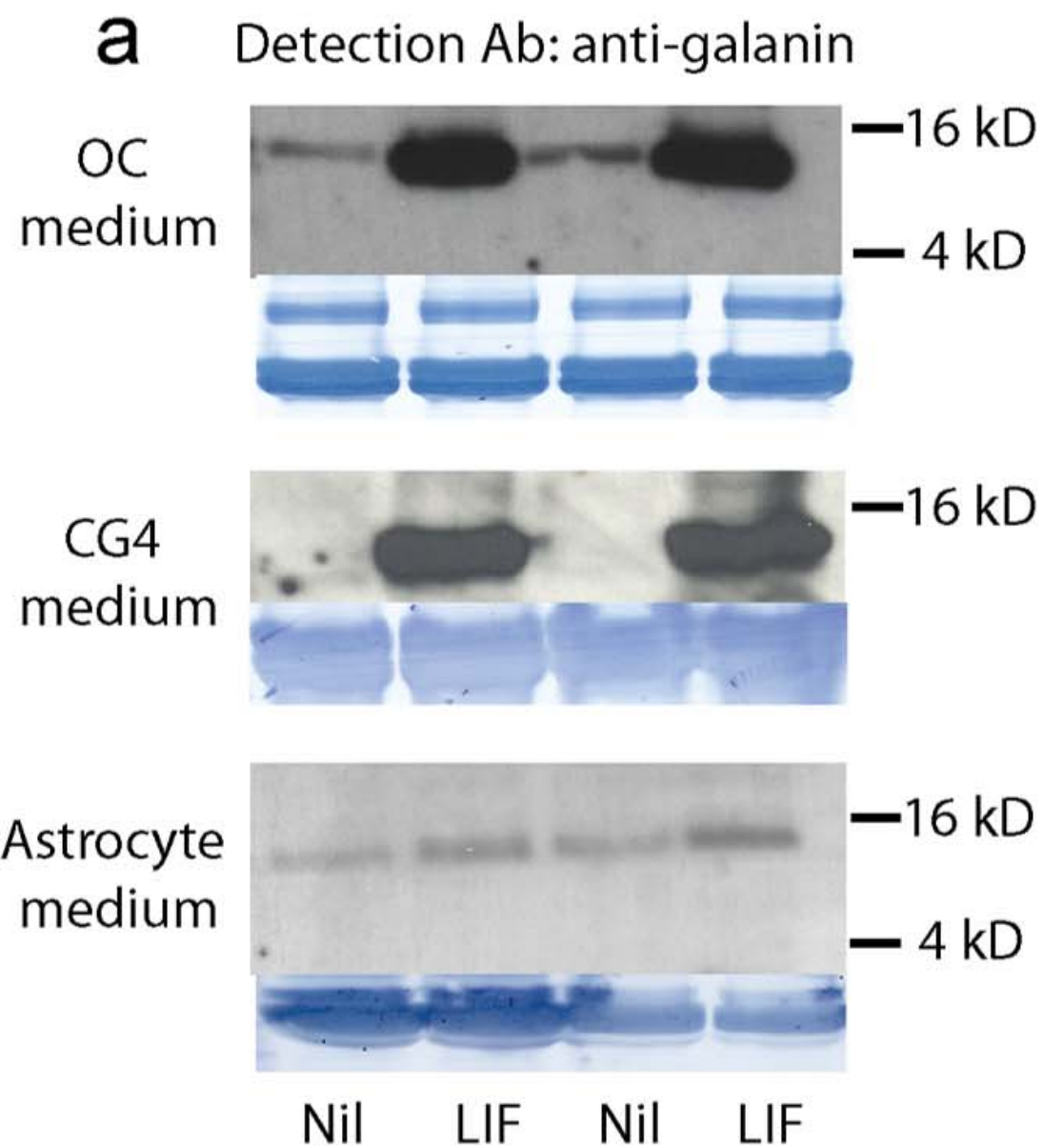
Table 3: Probe sets with differential expression between control and Insulin treatment ($p < 0.01$ and fold change > 2). Positive fold changes indicate increased expression in control samples compared to treated samples. Probeset ID refers to Affymetrix probe sets.

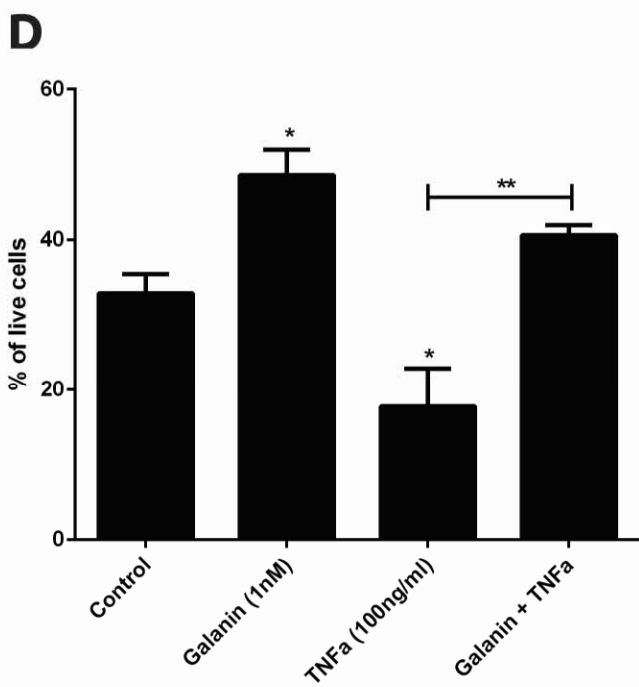
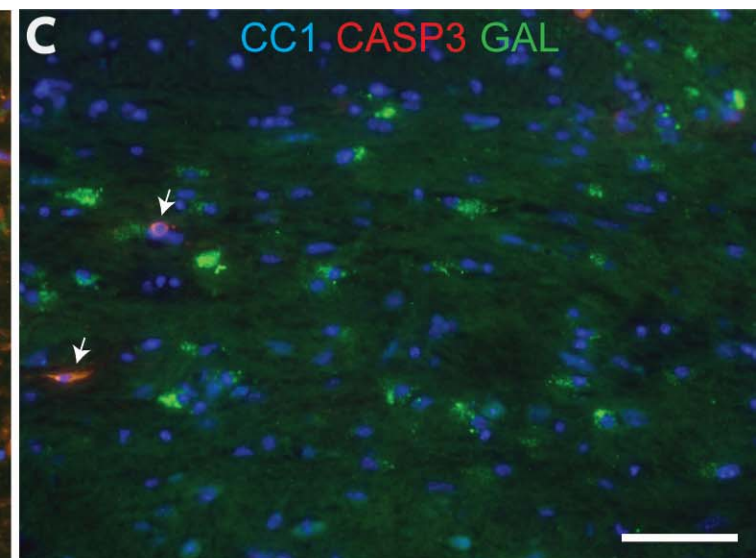
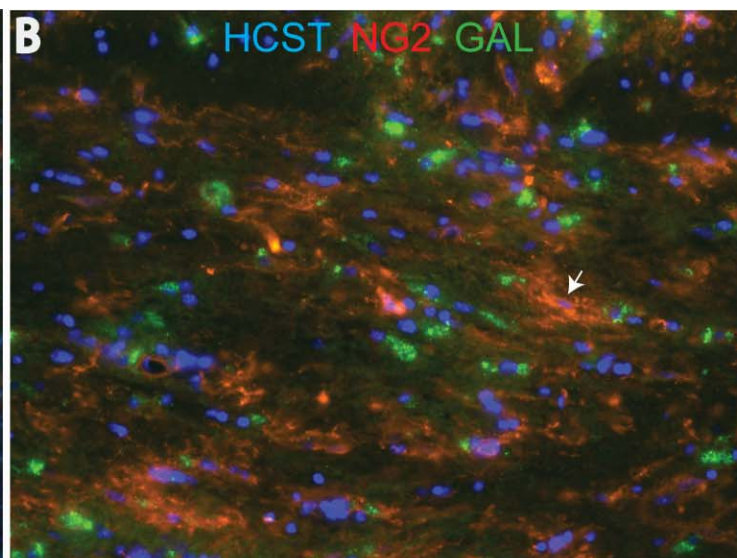
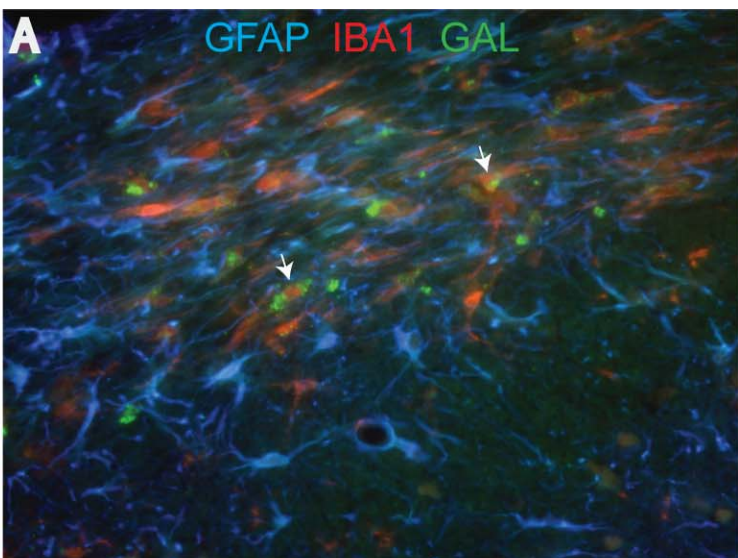
Figure S1: Galanin co-labeling studies, and oligodendrocyte tumor necrosis factor alpha (TNF α) survival assay. In curpizone treated mouse corpus callosum, we show that galanin (A, green) does not co-label with glial fibrillary acidic protein positive astrocytes (A, GFAP, blue), but does co-label with a few IBA-1 positive microglia/macrophages (A, red, arrows). Galanin (green) was not found to co-label with NG2 positive oligodendrocyte lineage cells (B, red, arrow), or activated caspase 3/CC-1 double positive oligodendrocytes (C, red and blue respectively, arrows). In D, exogenous galanin was found to promote the survival of oligodendrocytes derived from CG4 cells in the presence of TNF α . Cells were seeded at a density of 5000 cells/well in an 8 well chamber slide, and exposed to galanin (1nM), TNF α (100ng/ml), or galanin and TNF α for 72 hours. For live/ dead cell counts, 5000 cells/field (4 fields per condition), in 3 independent experiments, were assessed. Data are presented as mean percentage of live cells \pm SEM (* $P < 0.05$, ** $P < 0.01$, ANOVA).

WT vs Gal KO OC survival

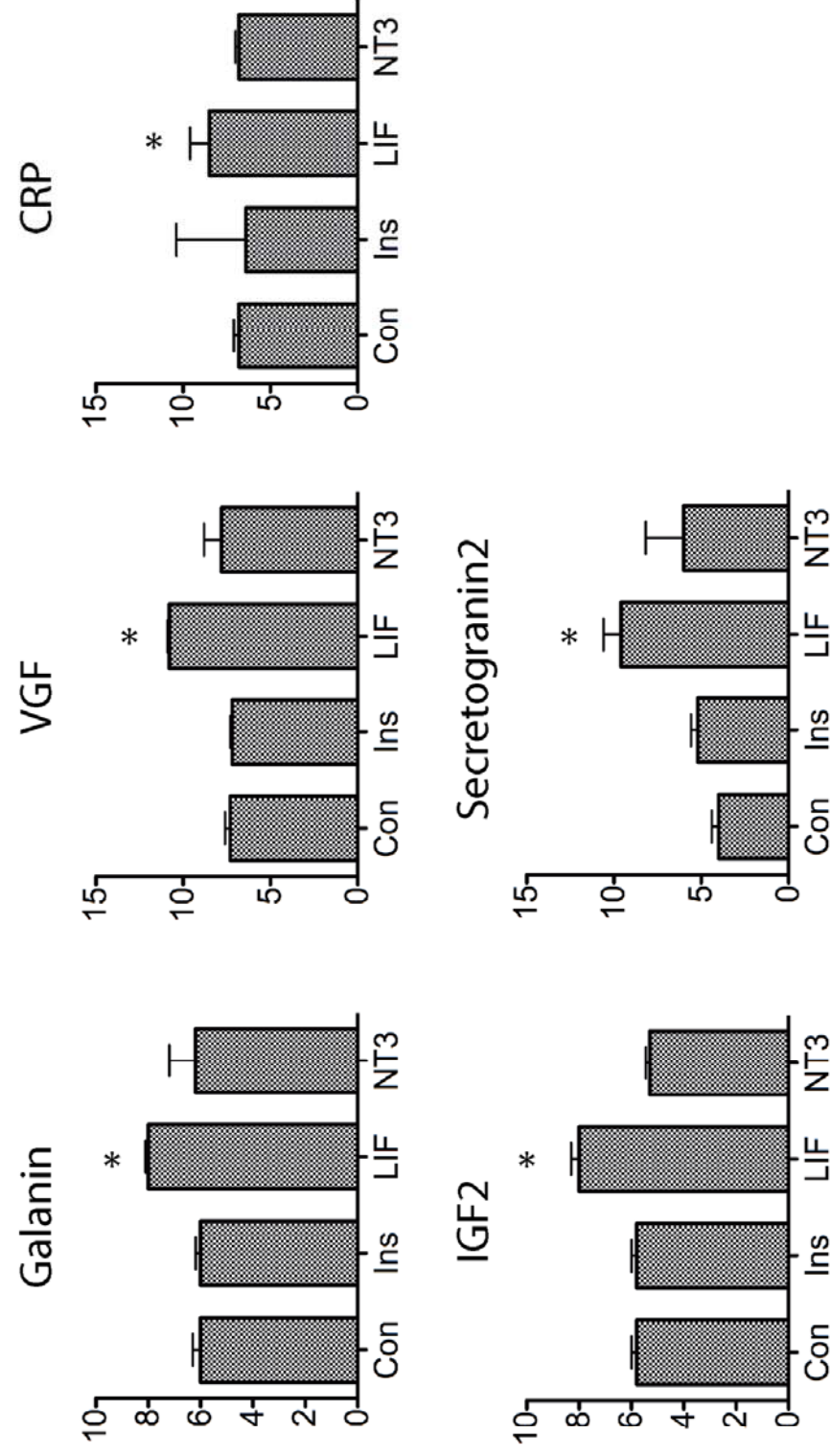




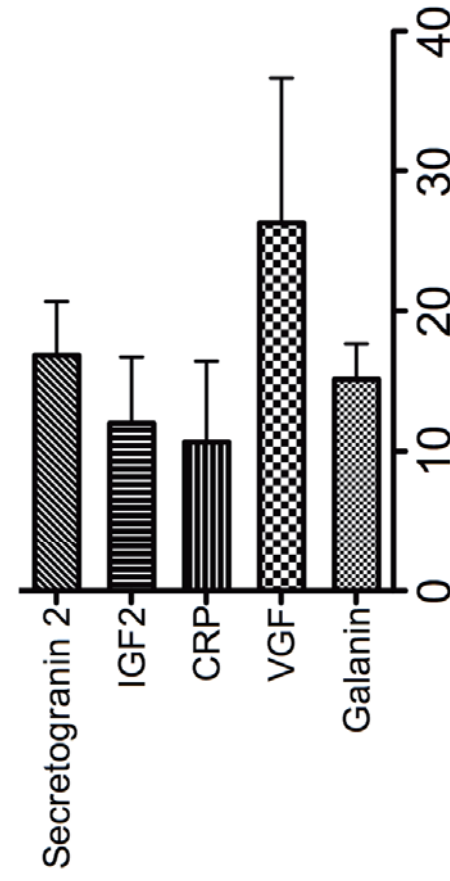




a



b



LIF-induced relative gene expression (fold change)